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Production of alkaline protease from halotolerant *Bacillus* sp. through submerged fermentation and study of its fermentation kinetics

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ABSTRACT

In this study, several salt-tolerant bacteria were isolated from the mangrove ecosystem of Bhitarkanika, Odisha. Further, the strains were screened for their protease production in skim milk agar medium. Among the various bacterial isolates, BSB6 and BSB12 exhibited a comparatively larger hydrolyzing zone on solid medium. Both strains were characterized morphologically, physiologically, and biochemically. Both the strains found Gram positive motile rod and identified as *Bacillus* sp. The bacterial strains used in this study were *Bacillus* sp. The strains were isolated from the mangroves of Bhitarkanika, Odisha, and identified through phenotypic and biochemical characterization. A preliminary screening was conducted to assess the ability of *Bacillus* sp. to produce protease. The hydrolyzing capacity of representative *Bacillus* sp. strains was qualitatively assessed based on the formation of halo zones around the colony. Both isolates were grown in broth medium, and their extracellular protease activity was evaluated through submerged fermentation. Among the various incubation periods tested for protease production in submerged fermentation (SmF), the highest protease yield (1893.45±83.45 U/ml) was achieved on the 11th day when *Bacillus* sp. BSB6 was inoculated in the fermentation medium. The study concluded that the highest biomass of *Bacillus* sp. BSB6 (15.24±0.19 g/50ml) was achieved after 15 days of incubation. Similarly, maximum protease production (2556.97±46.52 U/ml) was observed on the 11th day when *Bacillus* sp. BSB12 was cultivated in the fermentation medium.

KEYWORDS

Alkaline Protease; Halotolerant *Bacillus* sp.; Submerged Fermentation; Fermentation Kinetics; Microbial Enzyme Production; Cell Immobilization

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Introduction

Proteases are essential industrial enzymes, comprising approximately 60% of the total enzymes used in various industries. These enzymes have significant applications in biotechnology and are extensively utilized in various industries, including tanning, biological detergent production, meat tenderization, peptide synthesis, food processing, pharmaceuticals, and bioremediation [1,2]. Proteases are found in plants, animals, and microorganisms. Neutral proteases primarily originate from plants. Proteases that exhibit optimal activity at a pH of 8 or higher are categorized as alkaline proteases and are typically produced by microorganisms. Proteases are commonly found in nature, and microbes are a preferred source due to their rapid growth, minimal cultivation space requirements, and simple steps of genetic modifications. This allows for the development of new enzymes with properties supported for various applications [3]. *Bacillus* is known to produce various extracellular enzymes, including proteases. These enzymes are regarded as "green chemicals" due to their environmentally friendly nature and have diverse applications, ranging from industrial processes to household products.

Microbial proteases are categorized into acidic, neutral, and alkaline types based on the pH level at which they exhibit peak activity. Alkaline proteases are particularly significant due to their strong proteolytic activity and stability across various alkaline environments. Most commercially available alkaline proteases are primarily produced by bacteria, with *Bacillus* species being the predominant source [2]. Their non-pathogenic nature and ability to thrive in a basic culture medium make them suitable for industrial applications [4]. Several species of *Bacillus* known for their role in protease production include *B. cereus*, *B. stearothermophilus*, *B. mojavensis*, *Bacillus* sp., and *B. subtilis* [5]. Proteases, also referred to as peptidyl or peptide hydrolases (E.C.3.4.21-24 and 99), are valuable enzymes in various industries. They function by breaking down peptide bonds within protein molecules through hydrolysis [6]. These enzymes constitute one of the three largest categories in the industrial enzyme sector, making up around 60% of global enzyme sales. They also dominate the industrial enzyme market worldwide.

This study aimed to screen and produce protease using both free and immobilized cells of *Bacillus megaterium* (MTCC 9204, BSB-6; MTCC 9205, BSB-12).

Materials and Methods

Microorganisms and culture maintenance

This study utilized the bacterial strains *Bacillus megaterium* BSB6 and BSB12. The bacterial strains were obtained from the mangrove ecosystem of Bhitarkanika, Odisha, and identified through phenotypic analysis and 16S rRNA gene sequencing [7]. Partial sequencing of the bacterial strains was conducted at IMTECH, Chandigarh. The strains were then deposited in the Culture Collection Centre and assigned Accession Numbers MTCC 9204

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(BSB6) and MTCC 9205 (BSB12). These 16S rRNA gene sequences are deposited in GenBank with accession codes FJ853653 and FJ973576. Currently, the strains are preserved in the Microbiology Laboratory at the Department of Applied Microbiology, MITS School of Biotechnology, Bhubaneswar, Odisha.

Subculture of the strains

In this study, the bacterial strains were cultured on nutrient agar plates using the streak plate method. The plates were then incubated at 37°C for 12 to 24 hours to allow colony growth. To ensure the purity of the strains, they were repeatedly streaked on fresh nutrient agar (NA) plates. The purified strains were preserved on nutrient agar slants at 4°C and sub-cultured every two weeks to maintain viability.

Screening of *Bacillus megaterium* MTCC 9204 (BSB6) and MTCC9205 (BSB12) for enzyme production

The bacterial strains BSB6 and BSB12 were spot-inoculated onto a skim milk agar medium and incubated at 35°C for 48 hours. Following incubation, the plates were treated with an $HgCl_2$-HCl solution for five minutes. Alkaline protease production was assessed by culturing the organism at pH 10.0.

Cultivation of *Bacillus megaterium*

It was cultivated in 250 mL Erlenmeyer flasks containing 25 mL of culture medium at 35°C for 2 days. The composition of the culture medium (g/L) in distilled water were: casein (10.0), glucose (10.0), yeast extract (3.0), peptone (5.0), NaCl (5.0), $CaCl_2{\cdot}2H_2O$ (0.4), and $MgCl_2$ (0.2).

Preparation of Crude Protease

After fermentation was completed, the enzyme was extracted from the fermented medium (SmF) following a modified version of the method described by Sethi et al. [8]. To extract the enzyme, 50 ml of cold 0.1 M phosphate buffer (pH 6.5) was added to the solid substrate cultures, and the mixture was manually shaken for 30 minutes at 30°C. The resulting mixture was then filtered using cheesecloth and centrifuged at 8,000 rpm for 15 minutes at 4°C to separate the cells and other insoluble materials. The supernatant obtained was further purified by filtering it three times through Whatman No. 1 filter paper, and the resulting filtrate was collected as the crude enzyme.

For enzyme immobilization, the harvested beads were cooled at 4°C for an hour. Following this, each filtrate underwent centrifugation at 8,000 rpm for 30 minutes at 4°C using an ultracentrifuge (Remi Compufuge CPR-24). The final supernatant obtained was used as crude protease for enzyme assay [9].

Determination of enzyme activity

Extracellular protease activity was assessed using the method described by van den Hombergh. For this, 450 µl of the sample was mixed with 50 µl of 1% (w/v) BSA (Fraction V, Sigma) in 0.1M sodium acetate buffer (pH 4.0) and incubated at 37°C for 30 minutes. The reaction was then halted by adding 500 µl of 10% (w/v) trichloroacetic acid (TCA). Following a 30-minute incubation at 0°C, the precipitated proteins were separated via centrifugation at 6000 rpm for 5 minutes. The absorbance of the TCA-soluble fraction was measured using the Lowry method.
Protease activity was measured in units (U), where one unit was defined as the enzyme quantity required to release 1 µg of tyrosine per minute under the specified assay conditions. The extracellular protease activity was represented in U/ml.

Determination of total protein content

The total protein content of the sample was measured using the Lowry et al. method, with bovine serum albumin (BSA) as the reference standard. To perform the analysis, 1 mL of diluted culture filtrate was combined with 4 mL of Biuret reagent and 1 mL of Folin-Ciocalteu reagent in a 3:1 (v/v) ratio. The mixture was thoroughly mixed and incubated at 37°C for 20 minutes. Following incubation, absorbance was recorded at 750 nm using a UV-visible spectrophotometer. The protein concentration was then determined by comparing the absorbance values with a standard curve generated using BSA.

Estimation of dry cell biomass

A pre-measured sample dry weight was used to calculate the biomass content. This was followed by centrifuging the sample for 15 minutes at 4°C at 8,000 rpm. After centrifugation, it was rinsed with sterile double-distilled water, and then filtered using Whatman No. 1 filter paper.

Later it was dried overnight in a hot air oven at 100°C achieving a constant weight. The dry weight of the cells was then measured and calculated using the formula by Sethi et al. [8].

Weight of biomass=weight of filter paper with biomass - weight of blank and dry filter paper

[Weight of biomass=weight of organism]

All experiments were conducted in triplicate, and the average value was taken as the final result.

Cell immobilization

Wet cells were obtained by culturing the organism in 250 mL Erlenmeyer flasks containing 100 mL of growth medium at 35°C for 48 hours. Following fermentation, the cells were collected by centrifuging the culture broth at 6000 RCF for 20 minutes at 4°C. The harvested cells were washed three times with sterile saline and re-centrifuged. These washed cells were then maintained at a uniform concentration for both immobilization and experiments involving free cells. To entrap the cells in calcium alginate beads, a syringe was used to extrude a mixture containing alginate slurry (3% w/v and 4% w/v) and wet biomass (0.6% w/v) into a chilled 0.2 M $CaCl_2$ solution at room temperature. The resulting beads were allowed to cure at 4°C for 1 hour. After curing, they were washed three to four times with sterile distilled water and stored for subsequent use.

Production of Protease by Repeated Batch Process

In the laminar airflow chamber, immobilized *Bacillus* sp. BSB6 and BSB12 cells were precisely filtered using Whatman No. 1 paper. Following two rounds of washing in a sterile saline solution (0.8% NaCl), the cells were kept in phosphate buffer (1M, pH 7.0) until further use. The beads were then moved into flasks with fresh growth media. After maximum amount of protease was produced, 50 milliliters of new fermentation medium was added to the spent medium, and the procedure was repeated several times until the beads started to decompose. At each cycle, cell leakage and enzyme activity were measured.

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Results

Isolation of Bacterial strain

In this study, a total of 12 bacterial strains were isolated from the mangrove soil of Bhitarkanika, Odisha, using Nutrient Agar Medium. The isolation process was carried out using both the pour plate and spread plate methods. The bacterial strains obtained were designated as BSB1 to BSB12. These strains were then individually screened on skim milk agar to assess their ability to produce protease. Among them, BSB6 and BSB12 formed halo zones in the medium, indicating protease production. These identified strains were subsequently preserved on Nutrient Agar Medium for further analysis.

Subculture of the strains for further use

The bacterial strains *Bacillus* sp. BSB6 and BSB12 were individually grown on Nutrient Agar (NA) plates. Their morphological characteristics were then examined. These strains, originally isolated from the mangrove ecosystem of Bhitarkanika, Odisha, were identified through phenotypic and biochemical analyses. To maintain their viability, the preserved bacterial cultures stored at -20°C were revived monthly on NA plates [Figure 1]. The sub-cultured plates of *Bacillus* sp. BSB6 and BSB12 are shown below.

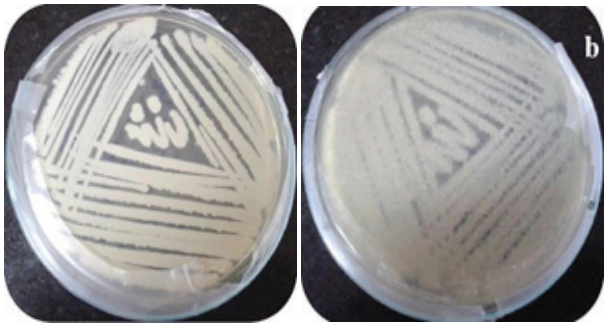

Figure 1. Sub-cultured plate of *Bacillus* sp. BSB6 and BSB12 (a,b).

Screening of bacteria for protease activity

A preliminary screening was conducted to evaluate the protease-producing ability of *Bacillus* sp. BSB6 (a) and *Bacillus* sp. BSB12 (b). The hydrolyzing capacity of these representative *Bacillus* strains was qualitatively assessed by measuring the halo zones formed around the colonies [Figure 2]. However, since the presence of clear zones on skim milk agar plates does not always directly correlate with actual protease activity, both isolates were further cultured in broth medium, and their extracellular protease activity was analyzed.

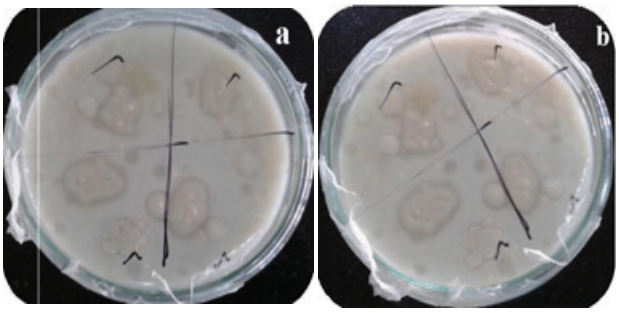

Figure 2. Screening of Bacillus sp. BSB6 and *Bacillus* sp. BSB12 (a, b).

Fermentation study

Submerged fermentation using *Bacillus* sp (BSB 6):

Among the various incubation periods tested for protease production in submerged fermentation (SmF), the highest yield (1893.45±83.45 U/ml) was observed on the 11th day when *Bacillus* sp. (BSB6) was introduced into the fermentation medium. After this point, protease production entered a stationary phase. Comparatively, the first day of incubation had the least impact on enzyme production. Additionally, the total protein content reached its peak on the 9th day of incubation in the protease produced by *Bacillus* sp. BSB6.

The total protein content in the crude extracts varied depending on the incubation period during fermentation. The highest protein content (6676.87±497.66 µg/ml) was observed in the crude enzyme extract when fermentation was conducted with gelatin on the 9th day of incubation. However, by the 11th day, the total protein content had decreased to 5397.69±26.80 µg/ml [Figure 3].

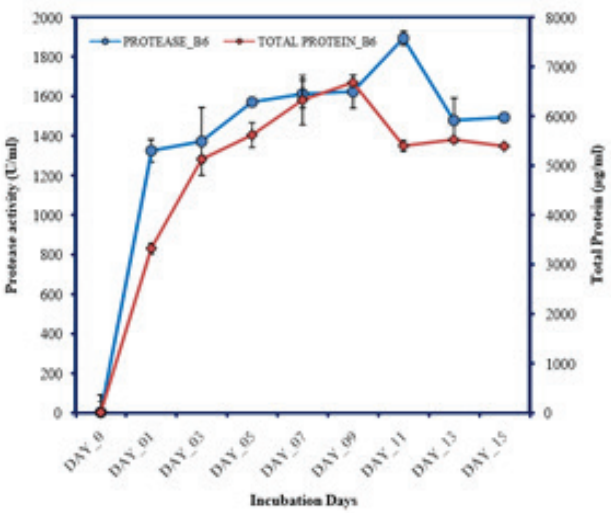

Figure 3. Production of protease using *Bacillus* sp. BSB6 at different incubation periods.

Biomass production during fermentation:

Bacillus sp. BSB6 cells were aseptically introduced into a submerged fermentation medium and incubated at 35°C for 15 days. Following fermentation, the biomass was carefully separated, processed, and measured. The findings indicated that the highest biomass yield of *Bacillus* sp. BSB6 was achieved after 15 days, reaching 15.24±0.19 g per 50 ml.

Submerged fermentation using *Bacillus* sp. (BSB 6):

Among the various incubation periods tested for protease production in submerged fermentation (SmF), the highest enzyme yield (2556.97±46.52 U/ml) was recorded on the 11th day when *Bacillus* sp. BSB12 was cultured in the fermentation medium. After this point, a stationary phase in protease production was observed. Comparatively, the lowest enzyme production (1578.52±28.9 U/ml) occurred on the 15th day of incubation. Additionally, the maximum total protein content was detected in the protease produced by *Bacillus* sp. BSB12 on the 9th day of incubation [Figure 4].

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The protein content in the crude extracts varied depending on the incubation period during fermentation. The highest protein concentration was observed in the crude enzyme extract when fermentation was conducted with gelatin on the 9th day of incubation, measuring 7598.43±49.30 µg/ml. However, by the 11th day of incubation, the total protein content had decreased to 6635.82±90.72 µg/ml [Figure 4].

Biomass production during fermentation:

Bacillus sp. BSB12 cells were aseptically introduced into a submerged fermentation medium and incubated at 35°C for 15 days. Following successful fermentation, the biomass was collected, processed, and measured. The findings of this study indicate that the highest biomass yield of *Bacillus* sp. BSB12 was achieved after 15 days, reaching 15.53±0.35 g per 50 ml.

Specific activity of protease:

Based on the collected data, the specific activity of protease was analyzed, revealing that *Bacillus* sp. BSB12 exhibited the highest specific activity (Table 1).

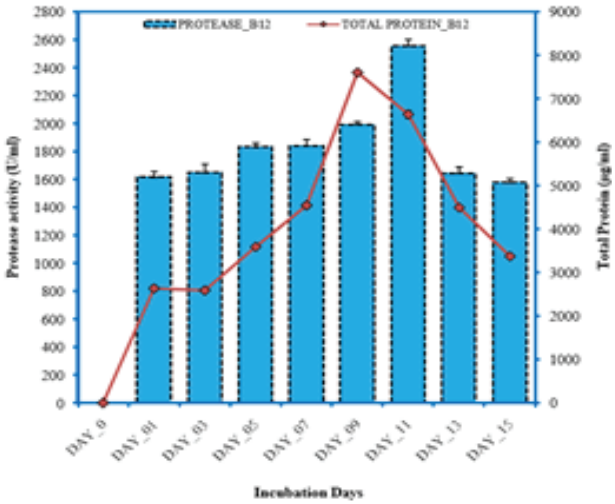

Figure 4. Production of protease using *Bacillus* sp. BSB12 at different incubation periods.

Table 1. Specific activity of protease produced by Bacillus sp. BSB6 and BSB12

Bacteria	Incubation period (days)	Protease activity (U/ml)	Total protein (mg/ml)	SA (U/mg)
Bacillus sp. BSB6	FIRST	1324.81±92.03	3.32±0.225	399.03
	THIRD	1371.61±56.35	5.12±0.109	267.89
	FIFTH	1570.63±171.46	5.61±0.072	279.96
	SEVENTH	1611.72±16.62	6.32±0.252	255.01
	NINETH	1623.40±70.88	6.67±0.497	243.38
	ELEVENTH	1893.45±83.45	5.39±0.026	351.29
	THIRTEENTH	1477.98±35.97	5.52±0.113	267.75
	FIFTEENTH	1492.65±112.74	5.38±0.028	277.44
Bacillus sp. BSB12	FIRST	1615.96±42.39	2.64±66.16	612.10
	THIRD	1648.61±62.29	2.58±27.95	638.99
	FIFTH	1832.40±28.95	3.59±22.30	510.41
	SEVENTH	1841.95±44.74	4.54±59.07	408.41
	NINETH	1994.43±20.81	7.59±49.30	262.77
	ELEVENTH	2556.97±46.52	6.63±90.72	385.66
	THIRTEENTH	1645.20±44.47	4.49±14.37	366.41
	FIFTEENTH	1578.52±28.19	3.37±44.02	468.40

Fermentation Kinetics

The fermentation kinetics parameters were assessed following the completion of protease production through fermentation. The yield parameters are presented in Table 2. The highest specific growth rates observed during the fermentation were 846.7 mg L^{-1} h^{-1} for *Bacillus* sp. BSB6 and 863.3 mg L^{-1} h^{-1} for *Bacillus* BSB12, respectively.

The rate of protease production (dP/dt) in the fermentation medium by *Bacillus* sp. BSB6 and *Bacillus* sp. BSB12 using free cells was observed to be 7.17 and 9.68 U mL^{-1} h^{-1}, respectively. When 3% immobilized cells were used, the dP/dt values were 9.02 U mL^{-1} h^{-1} for BSB6 and 6.63 U mL^{-1} h^{-1} for BSB12. For 4% immobilized cells, BSB6 and BSB12 exhibited dP/dt values of 8.65 and 9.49 U mL^{-1} h^{-1}, respectively. Overall, *Bacillus* sp. BSB12 demonstrated superior protease production efficiency compared to *Bacillus* sp. BSB6.

The growth-independent coefficient of enzyme production (β) was also higher when fermentation was conducted using both free and immobilized cells of *Bacillus* sp. BSB12.

Immobilization of cells of B. *megaterium* BSB6 and B. *megaterium* BSB12 for protease production

The cells of *Bacillus megaterium* BSB6 and BSB12 were immobilized using calcium alginate to produce protease, and their reusability was assessed. Immobilized cells were aseptically introduced into fermentation flasks for four consecutive batch cycles, using gelatin as the carbon source [Figure 5 and 6]. For BSB6 cells immobilized in 3% (w/v) alginate beads, protease activity increased gradually through the third cycle before declining in the fourth. When 4% (w/v) alginate beads were used for BSB6, protease production also increased from the first to the third cycle, peaking in the third cycle and remaining detectable through the fourth [Figure 7].

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Table 2. Fermentation kinetics of the protease produced by *Bacillus* sp. BSB6 and *Bacillus* sp. BSB12.

Strain & Condition	Ye/s (U/g)	Yx/s (g/g)	μ (h- 1)	β (U/g/h)	dx/dt (g/L/h)	dP/dt (U/ml/h)	X (mg/ml)	μmax (mg/L/h)
B6	189345	30.48	0.846	717.22	0.85	7.172	304.8	846.7
B6 (3%)	238042	---	---	901.67	---	9.02	---	---
B6 (4%)	228501	---	---	865.53	---	8.65	---	---
B12	255698	31.08	0.863	968.55	0.863	9.685	310.8	863.33
B12 (3%)	174938	---	---	662.64	---	6.63	---	---
B12 (4%)	341833	---	---	949.53	---	9.49	---	---

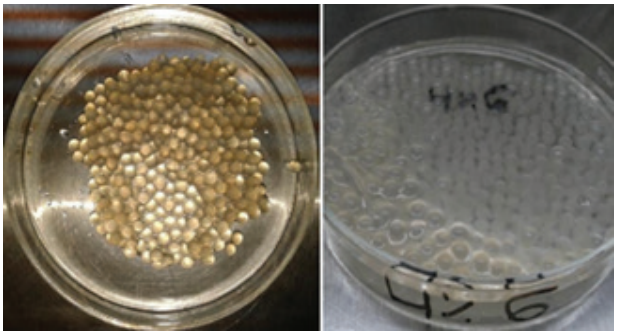

Figure 5. Immobilized cells of *B. megaterium* BSB6 (a: 3%, w/v and b: 4%, w/v).

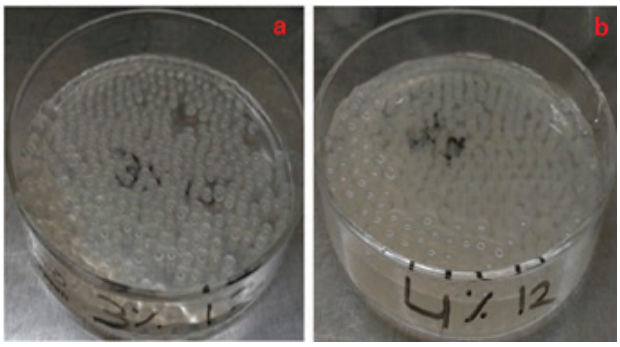

Figure 6. Immobilized cells of *B. megaterium* BSB12 (a: 3%, w/v and b: 4%, w/v).

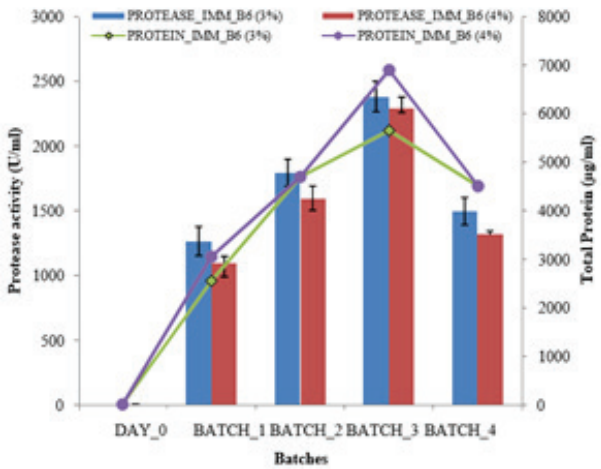

Figure 7. Production of protease using calcium alginate beads entrapping the cells of *Bacillus megaterium* B6 using repeated batch cultures performed at 30°C for 48h.

Meanwhile, the 4% (w/v) alginate beads began to disintegrate after the second cycle of fermentation. In contrast, the 3% (w/v) alginate beads entrapping *Bacillus megaterium* BSB6 cells remained intact until the late phase of the third fermentation cycle.

When 3% (w/v) alginate beads containing *Bacillus megaterium* BSB12 cells were used, protease activity showed a continuous increase over the first three reuse cycles, followed by a decline in subsequent cycles [Figure 8]. In contrast, with 4% (w/v) alginate beads, protease production steadily increased from the first cycle through to the final one. Notably, the highest enzyme activity was observed during the fourth batch when using 4% (w/v) alginate beads entrapping *B. megaterium* BSB12 cells (Figure 8).

In the case of *B. megaterium* BSB12, the beads began to disintegrate after the third cycle of fermentation when using 3% (w/v) alginate beads. However, the 4% (w/v) alginate beads encapsulating *B. megaterium* BSB6 cells remained intact even at the later phase of the fourth cycle [Figure 8].

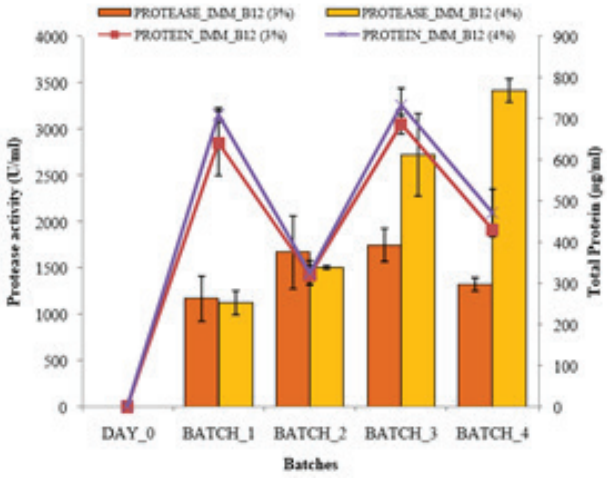

Figure 8. Production of protease using calcium alginate beads entrapping the cells of *Bacillus megaterium* BSB12 using repeated batch cultures performed at 30°C for 48h.

Discussion

In the present study, two halotolerant *Bacillus megaterium* strains (BSB6 and BSB12) were screened for their protease-producing capabilities using skim milk agar medium. Both strains demonstrated significant protease activity. A

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similar investigation by Ghasemi involved the screening of two moderately halophilic bacteria for protease production [10]. In that study, strains BSB6 (MTCC 9204) and BSB12 (MTCC 9205) were found to produce protease in broth medium by the 11th day of incubation. Among the two, BSB12 showed a higher protease yield (2556.97 ± 46.52 U/ml) compared to BSB6 (1893.45 ± 83.45 U/ml) at 37 ℃. While some researchers have reported an optimum temperature of 50 ℃ for protease production [11,12], others have found 37 ℃ to be optimal, particularly for strains such as *B. proteolyticus* CFR3001, *B. pumilus* SG2, and other *Bacillus* species [13].

The total protein content of the crude enzyme extracts varied depending on the incubation period during fermentation. The highest protein concentration (7598.43 ± 49.30 µg/ml) was observed on the 9th day of incubation when gelatin was used as the substrate. However, by the 11th day, the protein content had decreased to 6635.82 ± 90.72 µg/ml. The optimum incubation time for maximum protease production was found to be 48 hours, aligning with the findings of Shumi et al. (2004) for *Listeria monocytogenes* [14]. In contrast, maximum protease production was reported at 36 hours for *B. pumilus* SG2 [15] and 40 hours for *B. circulans* [16]

To enhance protease production and evaluate reusability, cells of *Bacillus* sp. BSB6 and BSB12 were immobilized using calcium alginate and subjected to repeated batch fermentation with gelatin as the carbon source [Figure 5 and 6]. When 3% (w/v) alginate beads containing *Bacillus* sp. BSB6 were used, protease activity increased with each reuse up to the third cycle, after which it declined. With 4% (w/v) alginate beads, protease production by *Bacillus* sp. BSB6 consistently increased from the first to the third cycle. For *Bacillus* sp. BSB12, the 3% alginate beads began to disintegrate after the third fermentation cycle. In contrast, the 4% alginate beads entrapping *Bacillus* sp. BSB6 remained intact even after the fourth cycle [Figure 8].

The enzyme yields of *Bacillus* sp. MTCC9205 reached its maximum at an inoculum size of 1%. These findings are consistent with those reported by Nagalakshmi and Ramesh for alkaline protease production by *Bacillus* sp., where strain MTCC2444 produced 699 U/L of the enzyme under similar conditions [13]. In the present study, a 1.5-fold increase in enzyme yield was observed when using immobilized cells compared to free cells.

Gel concentration plays a crucial role in enzyme production, with the optimal alginate concentration varying depending on the microorganism and the target product. To assess the effect of alginate concentration on the entrapment of *Bacillus* sp., three different sodium alginate concentrations were tested. Among these, 4% alginate yielded the highest production of alkaline protease [Figure 8]. In contrast, Idris and Suzana reported that a 2% gel concentration was optimal for lactic acid production from pineapple waste using immobilized *Lactobacillus delbrueckii* [17,18]. Higher gel concentrations can reduce enzyme yield due to restricted nutrient diffusion through the beads. Conversely, at 2%, the beads were too soft and fragile, leading to cell leakage.

Conclusion

In the present study, several salt-tolerant bacterial strains were isolated from the mangrove ecosystem of Bhitarkanika, Odisha. These isolates were subsequently screened for protease production using skim milk agar medium. Among them, strains BSB6 and BSB12 exhibited comparatively larger hydrolytic zones on solid medium, indicating higher protease activity. Both strains were characterized based on morphological, physiological, and biochemical traits and were identified as Gram-positive, motile rods belonging to the *Bacillus* genus.
Optimization of physical conditions for alkaline protease production by these *Bacillus* strains was performed, resulting in enhanced enzyme yields. The highest protease production (2556.97 ± 46.52 U/ml) was observed when *Bacillus* sp. BSB12 was cultured under optimized fermentation conditions. The findings of this study suggest that these moderately halotolerant *Bacillus* strains have significant potential for protease production, both in free and immobilized cell systems. The produced protease holds promise for direct application in various biotechnological processes.

Disclosure statement

No potential conflict of interest was reported by the author.

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